# Phylogenetic Differentiation of Wild and Cultured Sea Bream (*Sparus aurata*) Populations: 2. Genotypic Analysis

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**Abstract:** Stock identification aims to identify the subpopulations and several techniques may be used to this end. The aim of this study was to analyze and compare genetically between wild and cultured populations of sea bream using Microsatellite marker. 100 individuals from both sexes were randomly collected from 3 wild populations and one cultured population. The number of amplified bands detected varied, depending on the primers and population. The highest interpopulation genetic similarity (91%) exhibited between culture and Bardawil population and also between Bardawil and Suez Canal. While, the lowest genetic similarity (80%) was recorded between Alexandria and Suez Canal populations. Differences in genotype reflected the same amount of differences in phenotype among the studied populations. Phenotypic differences between populations can be taken as evidence of genetic differentiation. Finally, each of phenotypic or genotypic analysis can be used to classify fish populations with the same results up to the intraspecific level, or both of them can be used to assess the degree of phenotypic plasticity shown by populations.

***Keywords:*** Sea Bream, *Sparus aurata,* Phylogenetic Differentiation, Genotypic Analysis

## INTRODUCTION

Sea breams represent an essential aspect of the coastal marine ecosystem. Since the main goal is to maximize the efficiency of aquaculture production, the interest in genetic improvement studies rises. Molecular genetic techniques may contribute in classifying and defining the relationships among different species and local populations.

Molecular markers can be useful in escapee allocation since the fish farmed in Mediterranean form genetically distinct groups compared to their proximal wild population (Alarcón *et al.,* 2004; Karaiskou *et al.,* 2009; Loukovitis *et al.,* 2012). Microsatellite analysis can be used to identify fish farm escapees and to evaluate their potential genetic impact on wild populations.

In humpback whales, Palsboell *et al.* (1997) used microsatellite loci analysis to definite identification of individuals. Also, the genetic markers are permanent (Haig, 1998), traceable through further generations (Olsen *et al.,* 2000). The aim of this study was to use genotype analysis based on microsatellites (simple sequence repeat, SSRs) fingerprinting between wild (Bardawil, Suez Canal and Alexandria populations), and culture population (El-Deeba zone) of sea bream to help assess the degree of phenotypic plasticity shown by these populations.

## MATERIALS AND METHODS

The present study was carried out at Fish Production Laboratory, Animal Production & Fish Resources Department, Faculty of Agriculture and biotechnology laboratories, Suez Canal University.

**Collecting Samples:**

Randomly hundred individuals from both sexes, of each of different populations of sea bream **(***Sparus aurata***)** were collected from different environments; wild populations including [Mediterranean Sea (Alexandria beach), Suez Canal (Ismailia beach) and Bardawil lake] and cultured population (El-Deeba zone).

**DNA extraction:**

DNA extraction procedure of total genomic was performed according to the protocol of Genomic DNA Extraction using CTAB as follows:

- Grind 200 mg of fish tissue in approximately 500 µl of CTAB buffer.
- Transfer CTAB/tissue extract mixture to a microfuge tube.
- Incubate the CTAB/tissue extract mixture for about 15 min at 55 °C in a recirculating water bath.
- After incubation, spin the CTAB/tissue extract mixture at 12000 rpm for 5 min to spin down cell debris. Transfer the supernatant to clean microfuge tubes.
- To each tube add 250 µl of Chloroform: Iso Amyl Alcohol (24:1) and mix the solution by inversion. After mixing, spin the tubes at 13000 rpm for 1 min.
- Transfer the upper aqueous phase only (contains the DNA) to a clean microfuge tube.
- To each tube add 50 µl of 7.5 M Ammonium Acetate followed by 500 µl of ice-cold absolute ethanol.
- Invert the tubes slowly several times to precipitate the DNA. Generally, the DNA can be seen to precipitate out of solution. Alternatively, the tubes can be placed for 1 hr at -20 °C after the addition of ethanol to precipitate the DNA.
- Following precipitation, the DNA can be pipetted off by slowly rotating/spinning a tip in the cold solution. The precipitated DNA sticks to the pipette and is visible as a clear thick precipitate. To wash the DNA, transfer the precipitate into a microfuge tube

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containing 500 µl of ice cold 70% ethanol and slowly invert the tube. Repeat. (Alternatively, the precipitate can be isolated by spinning the tube at 13000 rpm for a minute to form a pellet. Remove the supernatant and wash the DNA pellet by adding two changes of ice cold 70% ethanol).

- After the wash, spin the DNA into a pellet by centrifuging at 13000 rpm for 1 min. Remove all the supernatant and allow the DNA pellet to dry (approximately 15 min). Do not allow the DNA to over dry or it will be hard to re-dissolve.
- Resuspend the DNA in sterile DNase free water (approximately 50-400 µl H2O; the amount of water needed to dissolve the DNA can vary, depending on how much is isolated). RNaseA (10 µg/ml) can be added to the water prior to dissolving the DNA to remove any RNA in the preparation (10 µlRNaseA in 10 ml H2O).
- After resuspension, the DNA is incubated at 65o C for 20 min to destroy any DNases that may be present and store at 4o C.
- Agarose gel electrophoresis of the DNA will show the integrity of the DNA, while spectrophotometry will give an indication of the concentration and cleanliness.

**PCR master mixture preparation:**

One PCR Master Mix (2X) (MB208-0100, Gene Dire X.) was used with specific primer and thermal cycler conditions according to (Launey *et al*., 2003; Brown *et al*., 2005) (Table 1).

**Inter-Simple Sequence Repeat (ISSR) amplification:**

Amplification of total genomic DNA through Polymerase Chain Reaction (PCR) system was formed using Thermocycler. PCR for amplified genomic DNA was carried out according to (Launey *et al*., 2003; Brown *et al*., 2005) with some modifications.

**Agarose gel preparation and detection of the amplification products using gel electrophoresis:**

Agarose Solution of concentration 1.5% was prepared by adding 1g agarose powder to 100ml of 1x TAE electrophoresis buffer in a conical flask. Insert this mixture into microwave to dissolve the agarose and make soluble mixture (liquid state). The agarose was cooled at room temperature for a minute. After inserting the comb in the tray, the agarose solution was poured in it. The agarose was poured carefully to avoid forming bubbles. The gel solidified within 15 min and became cloudy. The electrophoresis apparatus was filled with the TAE buffer and the comb was removed creating the wells for sample application. The samples were injected into the wells. After closing the cover of the electrophoresis, the power supply was connected. It was adjusted at 80 Volts for 100 min. The gel was removed from its bed and transferred to the gel staining tray for staining with ethidium bromide for 30 min followed by 20 min distain in distilled water.

**Genotype analysis:**

The amplified DNA fragments were separated on 1.5% agarose gel and stained with ethidium bromide. Fast Ruler Middle Range DNA Ladder (3000, 1500, 1000, 900, …, 100 bp) was used in this study. The amplified patterns were visualized on an UV transilluminator and photographed by gel documentation system. Gel documentation system (Geldoc-it, UVP, England), was applied for data analysis using Total lab analysis software, www.totallab.com (Ver.1.0.1).

**Table (1):** Illustrate Microsatellite Primer sequences, features and annealing temperature

| No. | Primer names | Sequence | Annealing temp. |
|---|---|---|---|
| 1 | Sal10 | F: TCACGGGGGACCAAGACTG<br>R: CTCACACTGCCTAATTAGCACAGA | 62 °C |
| 2 | Sal12 | F: ACGGTATGGAGTCAACTGC<br>R: CCCCTTTTGGTACATCATAG | 60 °C |
| 3 | Sal14 | F: TGCCAGAATGAATACCAACTGGTG<br>R: ATAATCAAAGTACCCCTGCATGTC | 60 °C |
| 4 | Sal15 | F: ACACTGTCTTTCTGTCCCTCACAC<br>R: GAGTAACACAGCCTCAGTTGAAGC | 62 °C |
| 5 | Sal21 | F: GGACGCCACACCATGTTCA<br>R: AACCGAAGCTGATTGTTAGTGTGA | 60 °C |
| 6 | SauD69INRA | F: CGTTGATCCCTGAGAAGC<br>R: AATACACGGAGAGCCACTG | 58 °C |
| 7 | SauE82INRA | F: ATTGGGTGGCAGTTTAGTAGG<br>R: CACTGCGATGAGTGACCC | 58°C |
| 8 | SauH94INRA | F: GTCTGAATGTTCCCATAGCTC<br>R: GCCACAGCTGTAACTCACTC | 55 °C |
| 9 | SauI41INRA | F: AACAGTTTGTGATTATTCATCG<br>R: CACGTCTAACCTGTGATTAGC | 55 °C |
| 10 | SauK140INRA | F: TTTCACTGAGCTGGAGACTTG<br>R: AGAGTTGAGTCTGTTGCATGC | 60 °C |

ISSR patterns were analyzed and scored from photographs. For the analysis and comparison of the patterns, a set of distinct, well separated bands were selected. The genotypes were determined by recording the presence (1) or absence (0) in the ISSR profiles. Genetic similarity (GS) between individuals i and j was calculated according the formula given as (Nei and Li, 1979):

$$B_{ij} = 2\ N_{ij} / (N_i + N_j)$$

Where, $N_{ij}$ is the number of common bands observed in individuals i and j, and $N_i$ and $N_j$ are the total number of bands scored in individuals i and j respectively, with regard to all assay units. Thus, GS reflects the proportion of bands shared between two individuals and ranges from zero (no common bands) to one (all bands identical). Genotype differentiation among cultured and wild Nile tilapia populations based on ISSR fingerprinting was analyzed by means of hierarchical cluster analysis of the SPSS 23.0 (1999) software package. The dendrogram was constructed using the average linkage between groups, and the data matrix so generated was used for calculation of similarity matrix for all primers based on Jaccard's coefficients method (Jaccard, 1908).

## RESULTS AND DISCUSSION

**Genotype analysis:**

All DNA samples from different Gilthead Sea bream (*Sparus aurata*) populations were examined by using Inter-Simple Sequence Repeat (ISSR) fingerprinting. Ten primers were used to determine DNA fingerprinting diversity in the different Gilthead Sea bream (*Sparus aurata*) populations. All the different primers used in this work, produced different ISSR band patterns (Figures from 1 to 11). The number of amplified bands detected varied, depending on the primers and population. Moreover, to ensure that the amplified DNA bands originated from genomic DNA, and not from primer artifacts, negative control was done for each primer/population combination. No amplification was detected in the control reactions. All amplification products were found to be reproducible when reactions were repeated using the same reaction conditions (Figures from 1 to 10).

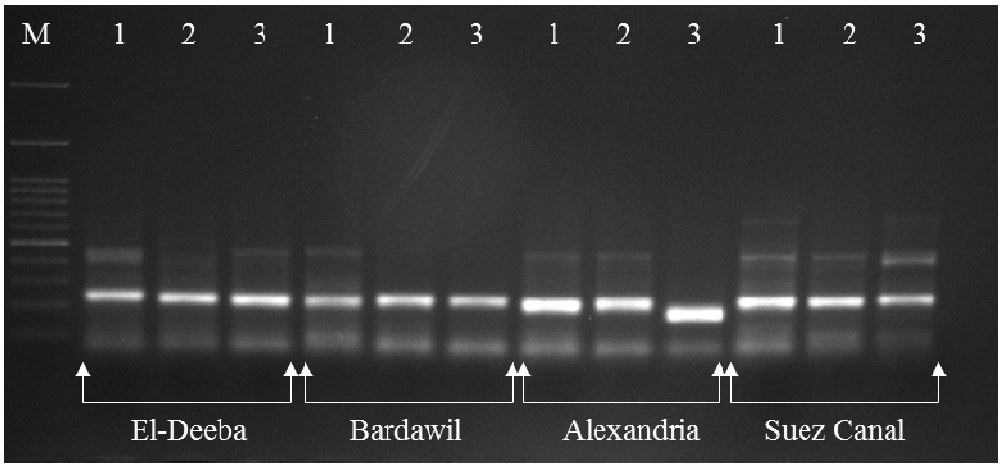


**Figure (1):** Patterns in different Gilthead Sea bream populations based on Sal10 primer (M: marker; 1,2, and 3 are replicates within each population)

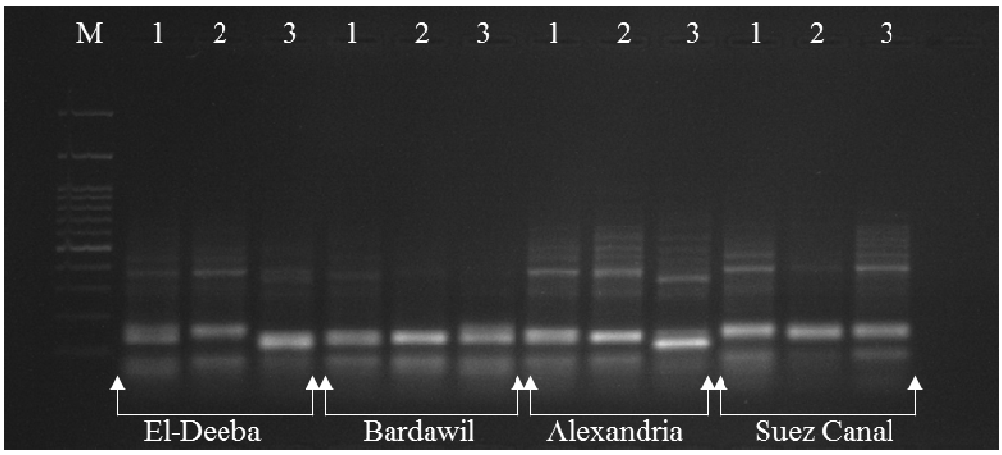


**Figure (2):** Patterns in different Gilthead Sea bream populations based on Sal12 primer (M: marker; 1,2, and 3 are replicates within each population)

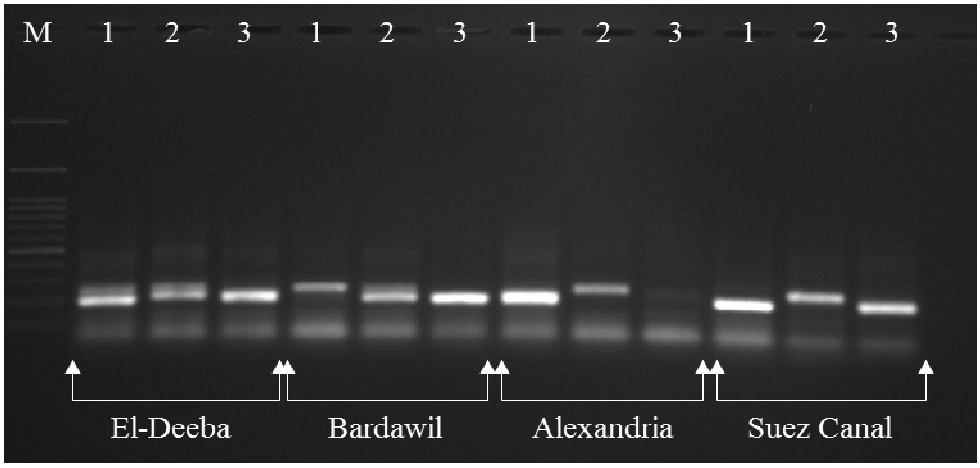


**Figure (3):** Patterns in different Gilthead Sea bream populations based on Sal14 primer (M: marker; 1,2, and 3 are replicates within each population)

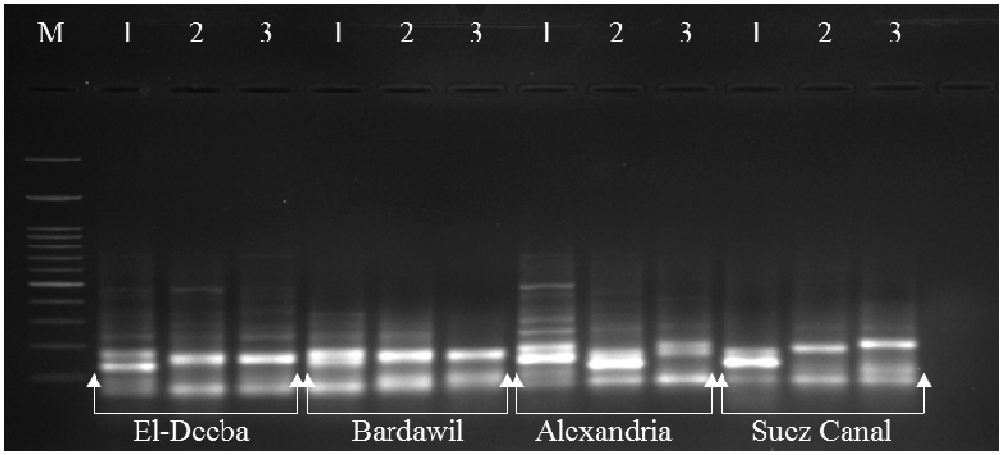


**Figure (4):** Patterns in different Gilthead Sea bream populations based on Sal15 primer (M: marker; 1,2, and 3 are replicates within each population)

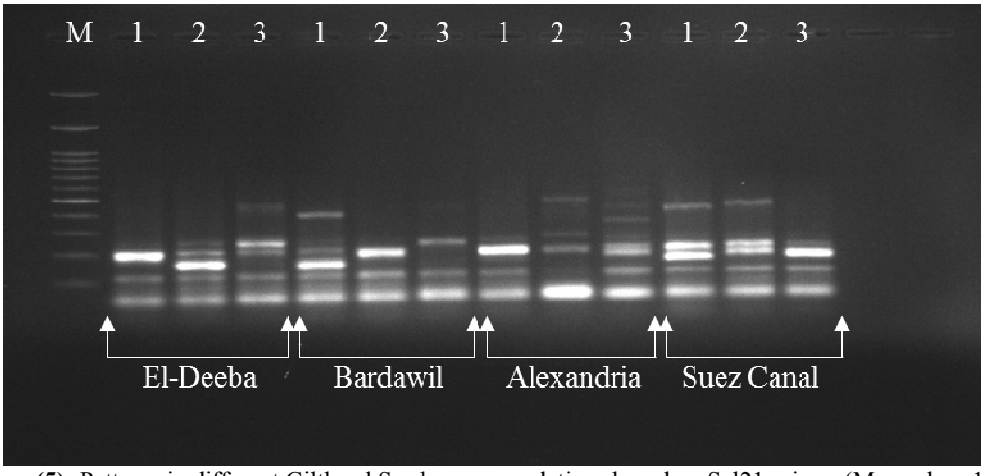


**Figure (5):** Patterns in different Gilthead Sea bream populations based on Sal21 primer (M: marker; 1,2, and 3 are replicates within each population)

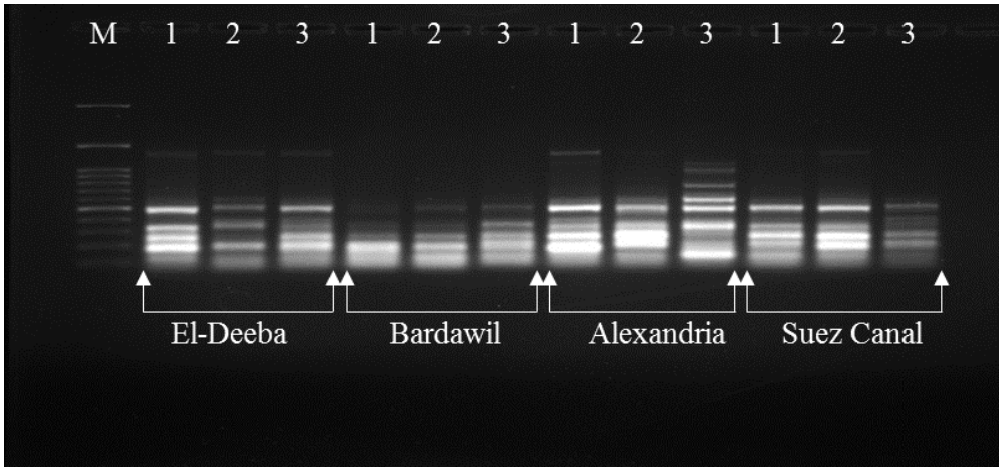


**Figure (6):** Patterns in different Gilthead Sea bream populations based on SauD69INRA Primer (M: marker; 1,2, and 3 are replicates within each population)

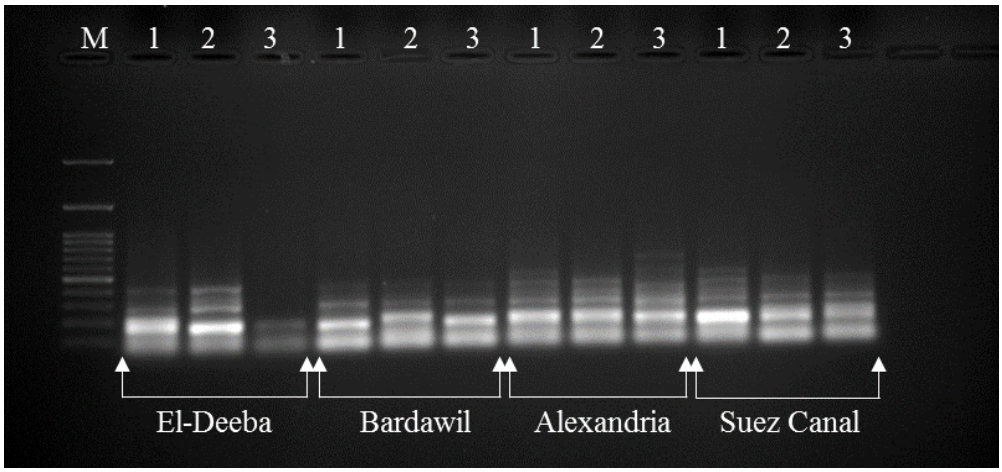


**Figure (7):** Patterns in different Gilthead Sea bream populations based on SauE82INRA primer (M: marker; 1,2, and 3 are replicates within each population)

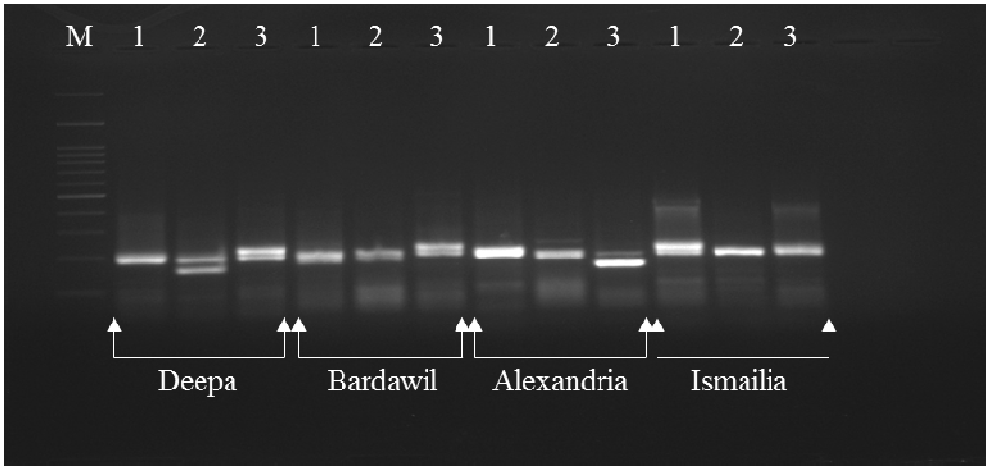


**Figure (8):** Patterns in different Gilthead Sea bream populations based on SauH94INRA primer (M: marker; 1,2, and 3 are replicates within each population)

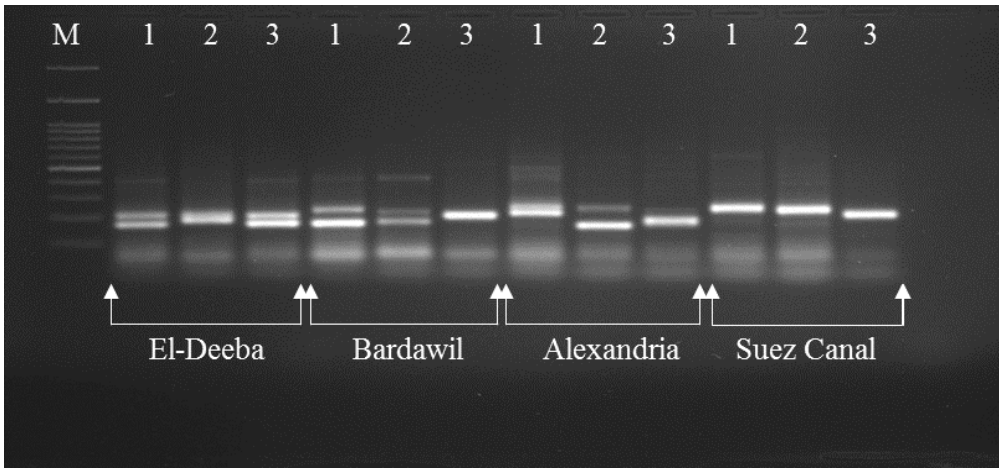


**Figure (9):** Patterns in different Gilthead Sea bream populations based on SauI41INRA primer (M: marker; 1,2, and 3 are replicates within each population)

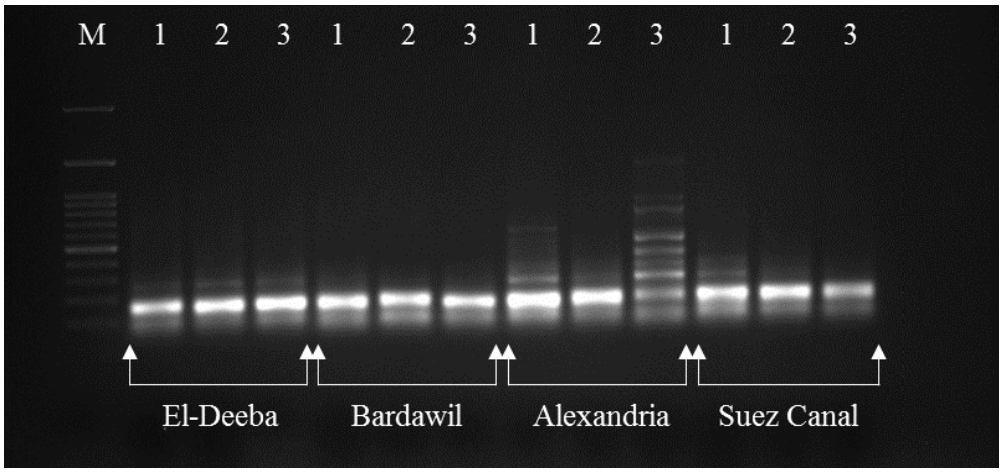


**Figure (10):** Patterns in different Gilthead Sea bream populations based on SauK140INRA Primer (M: marker; 1,2, and 3 are replicates within each population)

Data of genetic similarity coefficients among four populations of Gilthead sea bream (*Sparus aurata*) based on ISSR data of all primers used showed the highest interpopulation genetic similarity (91%) exhibited between culture and Bardawil population and also between Bardawil and Suez Canal, while the lowest genetic similarity (80%) was recorded between Alexandria and Suez Canal populations (Table 2).

Moreover, ISSR analysis was used for constructing phylogenetic tree illustrating the relationships among the different Gilthead sea bream (*Sparus aurata*) populations studied. The hierarchical cluster analysis based on ISSR fingerprinting, grouped the four populations into two clusters. Within these clusters, culture, Bardawil and Suez Canal populations were grouped close together while Alexandria population was grouped in a separated group. Also, a dendrogram showed that Bardawil population appears to be more genetically similar to that of Suez Canal population than that of culture population (Figure 11).

El-Zaeem and Ahmed (2006) stated that the identification of sex reversal and normal full-sib Nile Tilapia consider a great potential for detection the commercial deceit and protection of human health. RAPD analysis was applied to identify of six reversal and normal of full-sib Nile Tilapia. Eleven random primers were used to assay polymorphisms between these fish. The results showed that high polymorphic percentages (55.76%) were detected between sex reversal and normal full-sib Nile Tilapia.

The variations recorded in morphometric and landmark based on morphometric indices observed among striped red mullets population may reflect the environmental effects of the studied locations on growth and development of different body parts. These results are in accordance with the findings of (Akel, 1989) who reported differences in morphometric and meristic indices in Nile tilapia of different regions. Therefore, it was observed in this study that either the phenotype analysis based on a large number of morphometric character indices and meristic counts, or the genotype analysis based on ISSR fingerprinting can be used to discriminate fish populations with the same results up to the intraspecific level, or both the phenotype and genotype analyses can be used to assess the degree of phenotypic plasticity shown by different phenotypes.

The phylogeny of different Sea bream populations is considered problematic at the intraspecific level. Such a difficulty arises from highly homogeneous feature and morphology displayed by the different Sea bream populations and consequently, from the paucity of the key morphological characters suitable to address their phylogeny and evolution. In the present work, we have approached and compared the phylogenetic of different Sea bream populations based on each phenotype and genotype analysis. The results, presented by phenotypic (El-Zaeem *et al.,* 2020) and the present genetic dendrograms, proved that the amount of differences in genotype among the different Sea bream populations reflected the same amount of differences in phenotype among the same populations. Such phenotypic differences among these populations can be taken as evidence of genetic differentiation. Also, the results of genetic analysis confirmed the existing taxonomic system based on phenotype analysis. Moreover, the great concordance between each phylogeny based on phenotype and genotype analysis revealed that the phenotypic plasticity may not be found in the different Sea bream populations tested, and the relationship among them considered as intraspecific. Therefore, it was observed in this study that either the phenotype analysis based on a large number of morphometric character indices and meristic counts, or the genotype analysis based on ISSR fingerprinting can be used to discriminate fish populations with the same results up to the intraspecific level, or both the phenotype and genotype analyses can be used to assess the degree of phenotypic plasticity shown by populations.

**Table (2):** Genetic similarity coefficients among four population of Gilthead sea bream (*Sparus aurata*) based on ISSR data of all primers used

| Population | El-Deeba | Bardawil | Alexandria | Suez Canal |
|---|---|---|---|---|
| **El-Deeba** | - | 0.910 | 0.896 | 0.898 |
| **Bardawil** | - | - | 0.815 | 0.910 |
| **Alexandria** | - | - | - | 0.800 |
| **Suez Canal** | - | - | - | - |

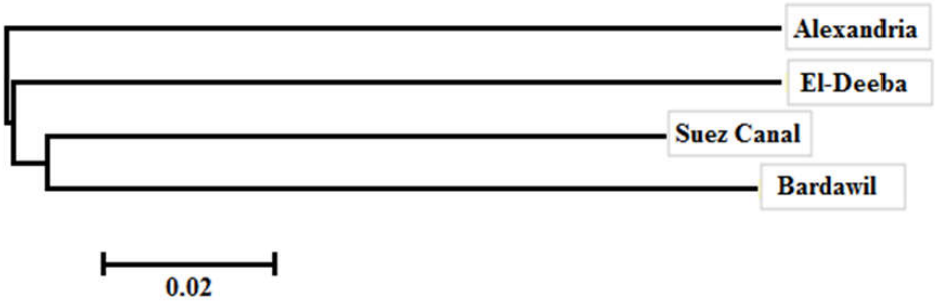


**Figure (11):** Dendrogram using average linkage (between groups) of different Gilthead seabream (*Sparus aurata*) population based on ISSR fingerprinting as shown by hierarchical cluster analysis.

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# التمييز في شجرة النشوء والتطور لعشائر أسماك الدنيس البرية والمستزرعة: 2. التحليل الوراثى

**يحيى سامى الزعيم[1] ، حافظ محمد خريبة[2] ، محمد سعد الدين الشريف[2] ، محمد فوزى الزرعى[2‡] ، منال محمد شاهين[2]**

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تهدف عملية كشف هوية السلالات إلي تحديد هوية العشائر الفرعية والتي يمكن أن يستخدم بها العديد من التقنيات. هذه الدراسة تهدف إلي التحليل والمقارنة الوراثية بين عشائر أسماك الدنيس البرية والمستزرعة باستخدام العلامات الوراثية من نوع الميكروستلايت. تم تمثيل كل عشيرة بعدد ١٠٠ عينة جمعت بشكل عشوائي من كلا الجنسين لهذا الغرض من أربعة عشائر ثلاثة برية (بحيرة البردويل – قناة السويس – الإسكندرية) وعشيرة من الأسماك المستزرعة (منطقة الديبة). حسب البريمر المستخدم والعشيرة اختلفت أعداد النقاط المضيئة المستكشفة. أعلى معدل للتماثل الوراثى بين العشائر (91%) تم ملاحظته بين عشيرة بحيرة البردويل وعشيرة الأسماك المستزرعة وأيضا بين عشيرة بحيرة البردويل وعشيرة قناة السويس. في حين أن أقل معدل للتماثل الوراثى بين العشائر (80%) تم ملاحظته بين عشيرة الإسكندرية وعشيرة قناة السويس. من خلال النتائج المتحصل عليها لوحظ أن الاختلافات في التركيب الوراثي تعكس نفس القدر من الاختلافات في النمط الظاهري بين العشائر موضع الدراسة. ومن هنا يمكن اعتبار الاختلافات المظهرية بين العشائر كدليل على التمايز الوراثى. أخيرًا، يمكن استخدام كل من التحليل المظهري أو الوراثى لتصنيف عشائر الأسماك بنفس النتائج حتى المستوى غير النوعي، أو يمكن استخدام كلاهما لتقييم درجة المرونة المظهرية التي أظهرتها العشائر.